

The University of Chicago

THE ENZYMATIC REDUCTION OF CYTOCHROME C (CYTOCHROME C REDUCTASE)

A DISSERTATION SUBMITTED TO THE
FACULTY OF THE DIVISION OF THE
PHYSICAL SCIENCES IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

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By ERWIN HAAS

No enzyme or enzyme system has thus far been described which catalyzes the physiological reaction between cytochrome *c* and reduced triphosphopyridine nucleotide. Although several of the known flavoproteins react with reduced triphosphopyridine nucleotide none of them reacts directly with cytochrome *c*. Cytochrome *b* and hydrogen carriers presumably acting between cytochrome *c* and the flavoproteins have therefore been postulated as part of this scheme but no concrete evidence to support such postulated mechanisms has been presented. The reactions between the reduced forms of either Warburg and Christian's "old" (1) or Haas' "new" (2) yellow enzyme and oxygen are too slow to be physiologically important and these enzymes are therefore left without explanation of their enzymatic significance. Theorell (3) reported observations of the reduction of cytochrome *c* by the "old" yellow ferment but this reaction also was too slow for physiological consideration. In the attempt to bridge the gap in the oxidation-reduction chain, Straub (4) isolated a flavoprotein from heart muscle, but like the other flavoproteins this substance does not react with cytochrome *c* (5).

In this paper we report the isolation of a new flavoprotein¹ which, reacting very rapidly with both oxidized cytochrome *c*

¹ In a preliminary report on the isolation of this enzyme we described it as colorless. During the first stages of its isolation the product lost much of its color upon purification. Judging from the activities of the previously isolated yellow ferments, we were led to the conclusion that the product could not be as active as it was and still show no yellow color. We were, therefore, misled by the extremely high activity of this enzyme.

and reduced triphosphopyridine nucleotide, completes the oxidation-reduction system from hexose monophosphate to cytochrome *c*. The prosthetic group of this new enzyme is alloxazine mononucleotide.

According to the nomenclature suggested by Warburg this enzyme could be designated as

alloxazine-proteid cytochrome *c* - triphosphopyridine nucleotide

We shall refer to it as cytochrome *c* reductase.

General Principles of Analytical System

The whole enzyme system which catalyzes the reduction of cytochrome *c* by hexose monophosphate is made up as follows: hexose monophosphate, *Zwischenferment*, triphosphopyridine nucleotide, cytochrome *c* reductase, cytochrome *c*. The triphosphopyridine nucleotide is rapidly reduced, while the oxidized cytochrome *c* does not react until the reductase is finally added. The concentrations of all components are so adjusted that (1) the rate of reduction of the cytochrome *c* is proportional to the reductase concentration and (2) the half time of the cytochrome *c* reduction is about 3 minutes. Each of the components of the system is sufficiently pure so that no reaction proceeds until the cytochrome *c* reductase is added.

Preparation of Test Substances

Triphosphopyridine Nucleotide—This substance was prepared by a modification of the method described by Warburg, Christian, and Griese (6). We are indebted to Dr. T. S. Ma for the following analysis of the final product.

$C_{22}H_{32}N_7P_3O_{19}$.	Calculated.	C 33.4, H 4.06, P 11.8
	Found.	" 34.8, " 4.37, " 9.3

On the basis of the phosphorus content we calculate a purity of 79 per cent for the triphosphopyridine nucleotide.

Using the manometric test for triphosphopyridine nucleotide (6) we found a purity of 80 per cent for our product.

Hexose Monophosphate—This was prepared by a modification of the directions given by Robison and Morgan (7) and by Warburg and Christian (1). We have found that bottom yeasts are

unsatisfactory for this preparation. Top ale yeasts, however, gave excellent results.

The best results are obtained when the Lebedew extract is prepared by digestion at 0° rather than at 35° as specified by Warburg and Christian. The yeast used must be fresh and it should be washed immediately after the brewing process. The extract should be protected from air during the extraction.

The yeast used in the preparation of the hexose monophosphate was the same as that used as the source of the reductase. 900 gm. of the dried powdered yeast were stirred into 2700 cc. of water and extracted for 22 hours at 0°. The air in the covered container was displaced by carbon dioxide. Centrifugation and filtration yielded 1.4 liters of the clear Lebedew extract, which was used at once for the preparation of hexose monophosphate. From 300 gm. of glucose, 300 gm. of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, and 20 gm. of KH_2PO_4 we obtained 42 gm. of the crystalline calcium salt of hexose monophosphate.

Analysis of this material by Dr. T. S. Ma gave the following result.

3.128 mg. sample	yielded	19.70 mg.	ammonium phosphomolybdate
3.416 " " "		1.240 " "	H_2O and 2.784 mg. CO_2
3.681 " " "		1.403 " "	" " 2.986 " "
Calculated, $\text{C}_6\text{H}_{11}\text{O}_5\text{CaPO}_4 \cdot \text{H}_2\text{O}$. H 4.11, C 22.8, P 9.8			
Found (average). " 4.13, " 22.18, " 9.15			

The calcium salt was converted into the potassium salt by treatment with potassium oxalate. 3.15 cc. of M oxalate were necessary for 1 gm. of the calcium salt. Thus 1 mole of calcium salt (mol. wt. 316) was equivalent to 0.995 mole of potassium oxalate.

Zwischenferment—The specific protein which is necessary in the reduction of triphosphopyridine nucleotide by hexose monophosphate was prepared in accordance with the directions given by Warburg and Christian (1). This is a relatively impure product but contained no cytochrome reductase, whereas the purest *Zwischenferment* obtained by the method of Negelein and Gerischer (8) contained some of the reductase.

Canadian top ale yeast which was used to make this preparation was washed and dried. A suspension of 400 gm. of the dried yeast in 1200 cc. of water was allowed to stand for 10 hours at

35°. The suspension was then centrifuged and the *Zwischenferment* precipitated from the supernatant solution by dilution with water and subsequent saturation with carbon dioxide. The precipitate, dried *in vacuo* over P_2O_5 , gave 6.4 gm. of a dry powder which has not lost its activity in more than 1 year's time. 0.1 mg. of this powder was necessary for each test.

Cytochrome c—Following the directions of Keilin and Hartree (9), we obtained from 5.8 kilos of horse heart 3.1×10^{-5} mole of cytochrome. The concentration was determined spectrophotometrically. Using the absorption coefficients given by Theorell (10), we found 5.17×10^{-7} mole of cytochrome per cc., while for the same solution, after being reduced, 5.15×10^{-7} mole per cc. of cytochrome was found. This result showed that the solution was free from colored impurities.

Isolation of Enzyme

The activity of an enzyme preparation was tested spectrophotometrically by the method to be described in detail in the next section. The protein concentrations were determined by evaporating the salt-free solutions. In the presence of salt the solutions were subjected to micro-Kjeldahl determinations for protein nitrogen. The protein concentrations were calculated on the assumption that all proteins present contained 16 per cent nitrogen.

The activity of each preparation is here calculated on the basis of mg. of dry protein. This activity (W) is defined as follows:

$$W = \frac{\Delta \log \text{oxidized cytochrome } c \text{ concentration}}{\Delta t \times \text{mg. protein}}$$

W is independent of the cytochrome c concentration. For the pure enzyme W has a value of $158 \text{ mg.}^{-1} \text{ min.}^{-1}$.

Source Material—Top ale yeast of the strain *Saccharomyces cerevisiae* I, Hanson, obtained from Drewry's, Ltd., was washed at 0° with tap water and then subjected to high pressure to remove the excess water. By blowing air over a thin layer of the yeast for a few hours it was thoroughly dried. The dried yeast can be stored at room temperature without apparent loss of activity.

Step 1. Extraction of Enzyme by Autolysis—4 kilos of the dry

yeast are suspended in 14 liters of water and kept for 33 hours at 20°. The suspension is centrifuged and 6.9 liters of clear solution obtained which contain most of the enzyme. The residue is washed with 6.5 liters of water and centrifuged. The resulting supernatant solution is combined with the original to give 13.8 liters of an enzyme solution which contained 1400 gm. of dry substance. $W = 0.27 \text{ mg.}^{-1} \text{ min.}^{-1}$. Since W for the pure enzyme is equal to $158 \text{ mg.}^{-1} \text{ min.}^{-1}$, the purity of the enzyme in the first extract is $0.27/158 = 0.0017$. The enzyme is very easily denatured and the whole of the following procedure must therefore be carried out at 0°.

Step 2. Fractionation with Ammonium Sulfate—At pH 4.5 the enzyme is soluble when the solution is 31 per cent saturated with ammonium sulfate. Under these conditions a large amount of inert proteins is denatured and can be removed. The enzyme is precipitated when the solution is made 51 per cent saturated with respect to ammonium sulfate.

Specifically, 13.8 liters of enzyme solution are cooled to 0°. While the solution is being stirred, 4.2 kilos of solid ammonium sulfate and then 220 cc. of 10 N acetic acid are added. The saturation is then 51 per cent with respect to ammonium sulfate, pH 4.5. The precipitate is separated by centrifugation. It contains the enzyme and can be kept in this form overnight. The precipitate, which has a volume of 1.1 liters, is suspended in 0.7 liter of water and 3 liters of a solution 31 per cent saturated with ammonium sulfate are added. The insoluble material is separated by centrifugation and discarded.

From the supernatant solution (4 liters) the enzyme is precipitated by adding 470 gm. of ammonium sulfate; the degree of saturation is then 51 per cent. After separation by centrifugation the enzyme is dissolved in 500 cc. of water. The solution contains 87 gm. of dry substance, other than ammonium sulfate. $W = 1.92$; purity after step (2) = 0.012; purification 7-fold; yield 44 per cent.

Step 3. Dialysis—For the following precipitation with alcohol it is necessary to remove the dissolved ammonium sulfate. Consequently the enzyme is dialyzed in cellophane tubes against running distilled water for 17 hours at 0°. After the removal of much inactive precipitate by centrifugation, 1200 cc. of enzyme

solution containing 52 gm. of protein are left. $W = 2.0$; purity = 0.0126; purification 1.06-fold; yield 63 per cent.

Step 4. Precipitation with Ethanol—1200 cc. of dialyzed enzyme solution are adjusted to pH 4.65 by adding 1.5 cc. of 2 N KOH. Slowly and with stirring in an ice bath, 600 cc. of 30 per cent cold ethanol are added. A fine white precipitate is formed which contains the enzyme. After 45 minutes the suspension is centrifuged and the precipitate is dried as rapidly as possible *in vacuo* over CaCl_2 at 0° . After this step 5.0 gm. of a dry powder are obtained. $W = 12$; purity = 0.076; purification 6-fold; yield 58 per cent. In 34 days 9 per cent of the activity of the dry powder is lost when kept at 0° *in vacuo* over CaCl_2 .

Step 5. Adsorption on Aluminum Hydroxide Gel; Elution with Alkaline Ammonium Sulfate—5.0 gm. of the enzyme powder are dissolved in 200 cc. of water. By adding 5 cc. of N KOH the pH is adjusted to about 9. Aluminum hydroxide gel γ prepared according to Willstätter and Kraut (11) is added fractionally until the supernatant solution is just colorless. Excess adsorbent should be avoided. About 7.0 gm. of aluminum hydroxide are usually necessary to adsorb all the enzyme.

The enzyme is eluted from the aluminum hydroxide by adding 50 cc. of a solution which is 64 per cent saturated with ammonium sulfate and is 0.1 N with respect to NH_4OH . This elution with alkaline ammonium sulfate is repeated twice. From the combined eluates (150 cc.) the enzyme is precipitated by adding 18 cc. of 2 N acetate buffer, pH 4.5, and 30 gm. of solid ammonium sulfate (degree of saturation, 70 per cent). The precipitate is centrifuged for 1 hour at 3000 times gravity to remove as much as possible of the ammonium sulfate solution which interferes in the following step. The enzyme is dissolved in 100 cc. of water and 4 cc. of N NH_4OH are added to adjust the pH to about 9. The solution now contains 360 mg. of protein. $W = 51$; purity = 0.32; purification 4.2-fold; yield 30 per cent.

Step 6. Adsorption on Calcium Phosphate; Elution with Phosphate—Tricalcium phosphate gel was prepared by following the direction of Utkin (12) and was used about 8 months after preparation. The tricalcium phosphate is added to the enzyme solution in small portions until the supernatant solution remains colorless. At this particular salt concentration about 6.5 gm. of

calcium phosphate are sufficient for adsorption of the enzyme. The tricalcium phosphate with the adsorbed enzyme is washed with 80 cc. of 0.02 M borate buffer, pH 9.2, and the enzyme is eluted with 40 cc. of 0.05 M phosphate buffer, pH 6.1. The elution is repeated two times and the eluates are combined. Excess of phosphate, which interferes in the next purification step, should be avoided. The 120 cc. of the phosphate solution contain 44 mg. of protein. $W = 98$; purity = 0.62; purification 1.95-fold; yield 24 per cent.

Step 7. Adsorption on Aluminum Hydroxide Gel Repeated—120 cc. of the phosphate solution are stirred for 10 minutes with 1.7 gm. of aluminum hydroxide and centrifuged. The colorless supernatant solution is discarded. The enzyme is eluted from the aluminum hydroxide three times with 12 cc. of alkaline ammonium sulfate as in step (5). To the 36 cc. of the eluate 7.5 gm. of solid ammonium sulfate and 4 cc. of 2 N acetate buffer, pH 4.5, are added. The degree of saturation with ammonium sulfate is 70 per cent. The precipitated enzyme is separated by centrifugation and dissolved in water. With 0.2 cc. of N NH_4OH the pH of the solution is adjusted to about 9. 17 mg. of enzyme dissolved in 6.2 cc. of solution are finally obtained. This solution is 0.2 M with respect to $(\text{NH}_4)_2\text{SO}_4$ but since a dilution of 1:7000 is made for the test there is no interference. $W = 138$; purity = 0.87; purification 1.41-fold; yield 55 per cent.

The titration with cytochrome as given in the next section proves that the enzyme after step (7) is 87 per cent pure. Although kept at 0° , the pure enzyme loses 30 per cent of its activity in 2 days time. Considering the instability of the enzyme, we believe that most of the inactive substance is cytochrome *c* reductase, denatured during the purification process.

Remarks Pertinent to Isolation—We have found cytochrome reductase in yeast obtained from different sources. The best results in the isolation are obtained with a yeast which autolyzes readily at low temperatures.

During the course of the isolation the purity of the enzyme increases from 0.0017 for the first step to 0.87 for the last step, an increase of about 500-fold. Assuming that the first extract contained all of the enzyme, we calculate that 1 kilo of dry yeast contains about 0.6 gm. of the enzyme ($0.0017 \times 1400/4$).

By the same method of calculation the amount of "old" yellow enzyme in Warburg and Christian's original source and the amount of "new" yellow enzyme in that of Haas have been calculated. These are here compared with the amount of cytochrome reductase found in our yeast (Table I).

It is interesting to note that the concentration of cytochrome reductase and that of the "old" yellow ferment are of the same order of magnitude.

The over-all yield for the seven steps of the isolation is equal to 0.63 per cent. By this process of purification about 235 kilos of dry yeast (approximately 1 ton of fresh yeast) would be necessary to obtain 1 gm. of the enzyme.

TABLE I
Yellow Enzymes in Yeast

Enzyme	Concentration, gm. enzyme per kilo dry yeast	Source material
Cytochrome <i>c</i> reductase....	0.6	Drewry's ale yeast
"Old" yellow enzyme.....	1.0	Schultheiss-Patzenhofer yeast
"New" " "	0.14	" " "

Purity of Final Product and Molecular Weight of Enzyme

In calculating the purity of the final product and in estimating the molecular weight we compare its properties with those of Warburg and Christian's "old" and Haas' "new" yellow enzyme.

Absorption Spectrum—The absorption spectrum from 250 to 550 $m\mu$ is given in Fig. 1, and the more detailed spectrum in the characteristic range 320 to 550 $m\mu$ is given in Fig. 2. The absolute values for the extinction coefficients² (ordinates) have been calculated with the assumption that the value at 455 $m\mu$ for cytochrome reductase is 1.04×10^7 $\text{cm}^2 \text{ mole}^{-1}$. This is the extinction coefficient of the "old" yellow enzyme (13) at 465 $m\mu$, of

² The extinction coefficient is defined by α in the relationship $I = I_0 10^{-\alpha c l}$ where c is expressed as moles per cc. The absorption coefficient β is defined by the equation $I = I_0 e^{-\beta c l}$. The value of β for the wave-lengths 455 $m\mu$ is then $2.4 \times 10^7 \text{ cm}^2 \times \text{mole}^{-1}$.

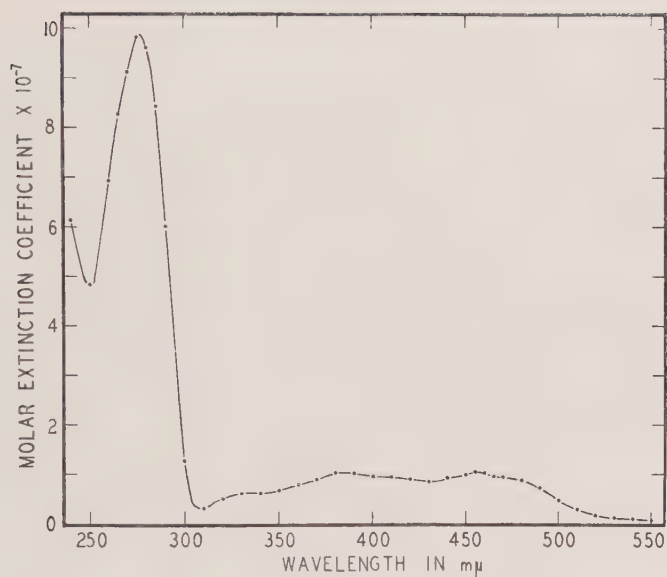


FIG. 1. Absorption spectrum of cytochrome *c* reductase

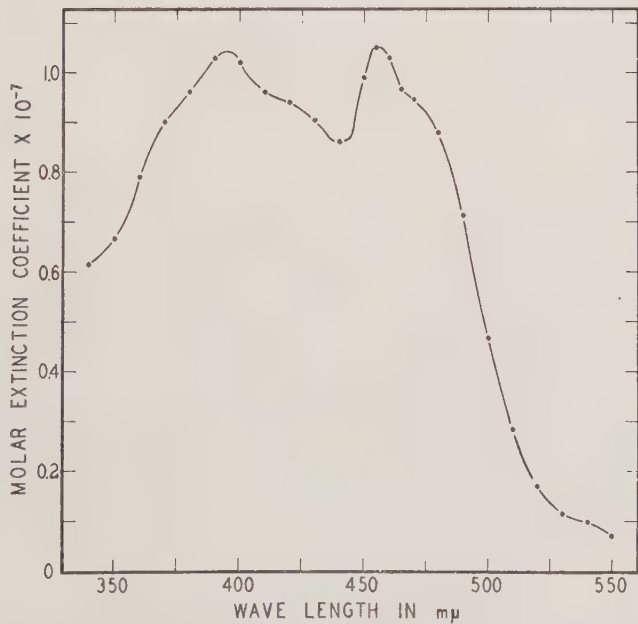


FIG. 2. Absorption spectrum of cytochrome *c* reductase, near ultraviolet and visible regions.

the "new" yellow enzyme (2) at 455 $m\mu$, and of the amino acid oxidase (14) at 450 $m\mu$.

The absorption spectrum of cytochrome reductase is made up of the three bands which are typical for the yellow enzymes, but as Table II indicates these bands are not identical with those of either the "old" or "new" enzymes.

The slight inflection in the absorption curve between 400 and 450 $m\mu$ indicates that a band at about 420 $m\mu$ may also be present. That this inflection is due to an impurity, very probably a hemin compound, was demonstrated by the fact that a band at this wave-length persisted when the cytochrome *c* reductase was reduced with the hexose monophosphate system. From the magnitude of this band we estimate that the maximum amount of hemin protein was 3 per cent. A small band also appeared at 557

TABLE II
Absorption Maxima of Yellow Enzymes

Enzyme	λ_1	λ_2	λ_3
	$m\mu$	$m\mu$	$m\mu$
Cytochrome reductase.....	275	385	455
"Old" yellow enzyme.....	275	380	465
"New" " ".....	275	377	455

$m\mu$. Although we have reason to believe this substance to be an impurity, its properties will be investigated further.

Determination by Light Absorption—With the assumption (1) that the extinction coefficient at the wave-length 455 $m\mu$ for cytochrome *c* reductase is 1.04×10^7 cm^2 mole⁻¹ and (2) that the enzyme is 100 per cent pure, we may calculate its molecular weight.

The enzyme solution obtained from step (7) in the isolation contained 2.75 mg. of protein per cc. For the same solution the light absorption, I_0/I , at 455 $m\mu$ was found to be 1.555 in an absorption cell 0.504 cm. in length. The molecular weight was then calculated in the following manner.

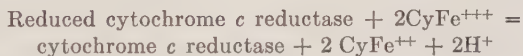
$$C (\text{flavin}) = \frac{1}{\alpha \times l} \log \frac{I_0}{I} = \frac{0.192}{1.04 \times 10^7 \times 0.504} = 3.66 \times 10^{-3} \text{ mole per cc.}$$

If 1 mole of the enzyme contains 1 mole of flavin, then the calculated molecular weight is $(2.75 \times 10^{-3}) / (3.66 \times 10^{-8})$ or 75,000.

Using a method similar to that employed here, Theorell (13) found a molecular weight of 73,000 for the "old" yellow enzyme. Kekwick and Pedersen (15) using the ultracentrifuge found by the equilibrium method a molecular weight of 82,800 for this same enzyme, while Polson (16) determined the molecular weight to be 77,800 by the sedimentation method. We can assume that within the limits of all the experimental errors the molecular weights of these two enzymes have the same value. In the following calculations we shall therefore assume the molecular weight of cytochrome *c* reductase to be 78,000.

From these same considerations we would arrive at the conclusion that our final product is a pure one but such a conclusion would be based on the assumption that the only yellow enzyme contributing to the light absorption was cytochrome *c* reductase. To determine its purity in this respect reduced cytochrome *c* reductase was titrated with cytochrome *c*.

Titration with Cytochrome c—Reduced cytochrome *c* reductase reacts with oxidized cytochrome *c* (CyFe^{+++}) according to the equation



Hexose monophosphate, *Zwischenferment*, and triphosphopyridine nucleotide were added to a solution containing the reductase. Excess hexose monophosphate was used but the amounts of *Zwischenferment* and triphosphopyridine nucleotide were such that the reduction took a relatively long time. After the reductase was reduced, an excess of oxidized cytochrome *c* was added. With relatively large concentrations the reaction between these constituents took place immediately and the amount of the cytochrome *c* reduced was determined spectrophotometrically at 550 $\text{m}\mu$. After the initial instantaneous reduction, the excess cytochrome *c* was reduced but this subsequent reduction was slow because of the limiting amount of *Zwischenferment*.

Determinations were made with two enzyme concentrations

TABLE III
Titration of Enzyme

$\lambda = 550 \text{ m}\mu$, total volume = 5.8 cc., length of cell = 1.98 cm.; gas space, nitrogen.

	Sample 1	Sample 2
0.025 M phosphate buffer, pH 7.3 . . .	5.8 cc.	
Hexose monophosphate	2.5 mg.	→
Triphosphopyridine nucleotide	0.007 mg.	
<i>Zwischenferment</i>	0.80 "	
Cytochrome reductase	0.52 "	1.04 mg.
Oxidized cytochrome <i>c</i> (added later)	4.7×10^{-3} mole	4.7×10^{-3} mole
Cytochrome reduced initially	1.18×10^{-8} "	2.32×10^{-8} "

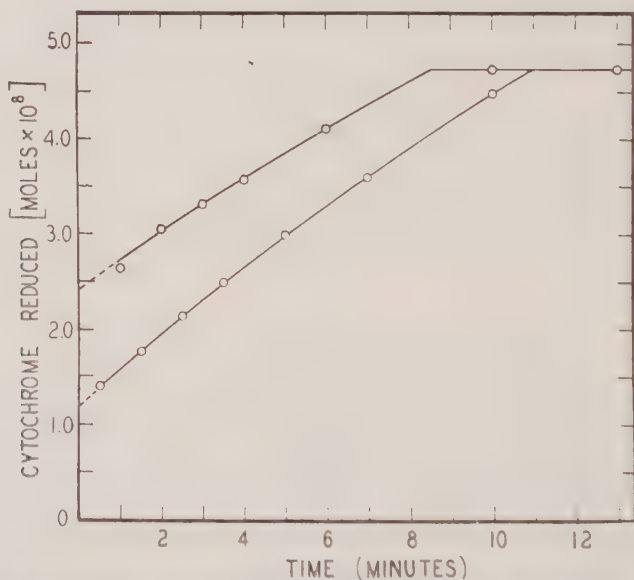


FIG. 3. Titration of reduced cytochrome *c* reductase, by oxidized cytochrome *c*. Upper curve 1.04 mg. of enzyme; lower curve 0.52 mg. of enzyme.

with identical results. The conditions under which these titrations were made are given in Table III and the results are shown in Fig. 3.

By extrapolating the curves to zero time, the amount of cytochrome *c* reduced instantaneously is obtained. The number of

moles of cytochrome *c* reduced in each case is given in the last row of Table III. Since 2 moles of cytochrome *c* are equivalent to 1 mole of cytochrome *c* reductase, we find that Sample 1 contains 0.59×10^{-8} mole of the reductase and Sample 2 contains 1.16×10^{-8} mole.

Assuming a molecular weight of 78,000 for the enzyme, Sample 1 contains $0.59 \times 10^{-8} \times 78,000 \times 10^3 = 0.46$ mg. of the active enzyme, while Sample 2 contains 90 mg. The purity of the enzyme preparation is given by the relation, (active enzyme)/(total protein) = $0.46/0.52 = 0.87$ for Sample 1 and $0.90/1.04 = 0.88$ for Sample 2.

On the basis of this titration and of the flavin determination in the previous section, we conclude that the enzyme was about 87 per cent pure and that inactive yellow enzyme accounted for most of the remainder.

The fact that identical results were obtained when different amounts of the reactants were used shows that the reaction goes to completion.

Stability of Enzyme

At pH 8.8 and at 0° the purest product in a 0.05 saturated $(\text{NH}_4)_2\text{SO}_4$ solution lost 30 per cent of its activity in 2 days. At pH 4.5 the enzyme is still less stable, losing as much as 50 per cent activity in 15 hours. When the enzyme solution was frozen by evaporation and then dried *in vacuo*, the dry powder lost only 30 per cent of its activity in 26 days if kept *in vacuo* over CaCl_2 at 0°.

The activity of the purified enzyme is destroyed by dialysis against distilled water. When dialyzed against 0.001 M NH_4OH , the enzyme is stable.

The inactivation of the enzyme by heat depends very greatly upon the salt concentration of the solution and upon the purity of the product.

At pH 7.3 in 0.06 M $(\text{NH}_4)_2\text{SO}_4$ the purest enzyme loses activity at the following rate: in 10 minutes, at 30° 19 per cent, at 40° 37 per cent, and at 45° 58 per cent.

The activity is destroyed by acetone and by dioxane.

Rudimentary Kinetics and Enzyme Test

Since the whole oxidation-reduction system consists of a series of steps, the rate of reduction of the cytochrome *c* may depend

upon the rate of any of these steps. We have not as yet separated these steps in making rate determinations; yet enough data are at hand to give minimum values for the rate constants of the reactions involving the oxidized and reduced forms of cytochrome *c* reductase. Under the conditions prevailing in our analytical experiments the rate of reduction is proportional to the concentration of the cytochrome *c* reductase. Since the reduction of oxidized cytochrome *c* involves the reduced form of the reductase, we may assume that all of the enzyme is present in the reduced form. Denoting oxidized cytochrome *c* by CyFe^{+++} we can express the over-all rate by the equation

$$-\frac{d(\text{CyFe}^{+++})}{dt} = K(\text{CyFe}^{+++})(\text{reduced cytochrome } c \text{ reductase}) \quad (1)$$

or

$$-\frac{d \log_{10}(\text{CyFe}^{+++})}{dt} = \frac{K}{2.3} (\text{reduced cytochrome } c \text{ reductase}) \quad (2)$$

Under these conditions the concentration of the reduced reductase remains constant during the course of the reaction and $d \log (\text{CyFe}^{+++})/dt$ is constant. This is demonstrated by the data given in Table IV.

The data given in Table IV are those used for determining the activity of our purest sample. All constituents except the cytochrome *c* reductase were added to the cell. After a few minutes were allowed for the triphosphopyridine nucleotide to become completely reduced, 0.00042 mg. of the reductase was added. The galvanometer reading was taken before adding the reductase and every minute thereafter. The value of the enzyme activity, W , for the final product was calculated to be $138 \text{ min.}^{-1} \times \text{mg.}^{-1}$.

$$W = \frac{\Delta \log(\text{CyFe}^{+++})}{\Delta t \times \text{mg. enzyme}} = \frac{0.058}{0.00042} = 138$$

By plotting the logarithm of the oxidized cytochrome concentration against the time of the reaction a straight line should be obtained and if Equation 2 is true the slope of the line should be proportional to the enzyme concentration. This plot is shown in Fig. 4 for three reactions with different reductase concentrations. By plotting the slopes of these curves against enzyme

concentration a straight line is obtained, in agreement with Equation 2 (Fig. 5).

The above considerations form the basis for the analytical test for the reductase. Under these conditions the rate of reduction is proportional to the enzyme concentration. A more thorough

TABLE IV
Test for Enzyme Activity

I_0 = light intensity (in arbitrary units) after passing through blank cell containing no cytochrome.

I = light intensity after passing through cell containing the cytochrome.

l = length of cell = 0.32 cm.

λ = 550 m μ .

α oxidized (for CyFe⁺⁺⁺) = $0.0956 \times 10^8 \text{ cm.}^2 \times \text{moles}^{-1}$.

α reduced (for CyFe⁺⁺) = $0.281 \times 10^8 \text{ cm.}^2 \times \text{moles}^{-1}$.

C = total cytochrome concentration = 5.4×10^{-8} mole per cc.

$$(\text{CyFe}^{+++}) = \frac{1/l \log I_0/I - \alpha \text{ reduced} \times C}{\alpha \text{ oxidized} - \alpha \text{ reduced}}.$$

Temperature 25°; gas space, air.

The following constituents were added, 1.0 cc. of 0.025 M phosphate buffer, pH 7.3, 0.90 mg. of potassium salt of hexose monophosphoric acid, 0.10 mg. of *Zwischenferment*, 0.020 mg. of triphosphopyridine nucleotide, and 0.86 mg. of cytochrome *c*.

Time	Galvanometer reading		$\frac{I_0}{I}$	(CyFe ⁺⁺⁺)	Log (CyFe ⁺⁺⁺) + 8	$\Delta \log (\text{CyFe}^{+++})$	$\frac{\Delta \log (\text{CyFe}^{+++})}{\Delta t}$
	I_0	I					
min.				moles per cc. $\times 10^8$			min. ⁻¹
0	180	123	1.47	5.40	0.732		
	Added 0.00042 mg. reductase						
1	180	111	1.62	4.70	0.672	0.060	0.060
2	180	103.5	1.74	4.14	0.617	0.115	0.058
3	180	96	1.87	3.62	0.559	0.173	0.058
4	180	90	1.99	3.16	0.500	0.232	0.058
5	180	85.5	2.10	2.76	0.440	0.292	0.058
6	180	80.5	2.23	2.36	0.373	0.359	0.060

study of the kinetics of this system will be presented in a later report.

The reaction between cytochrome *c* and cytochrome *c* reductase is first order with respect to each of these constituents. Since the cytochrome *c* undergoes a 1 valence change and the reductase a

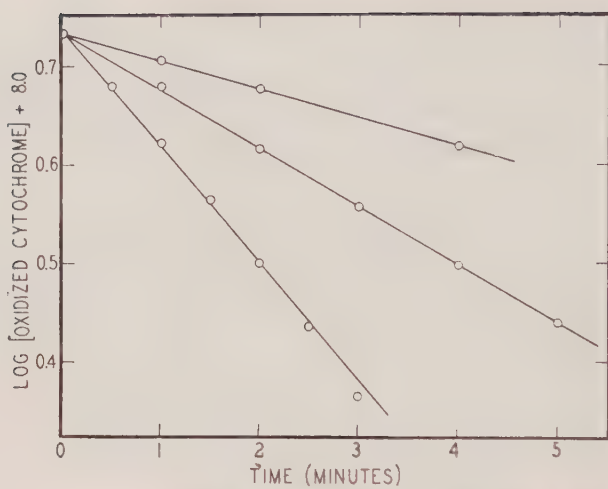


FIG. 4. Rate of reduction of cytochrome *c*. Top curve, 0.21 γ of reductase; middle curve, 0.42 γ ; bottom curve, 0.84 γ .

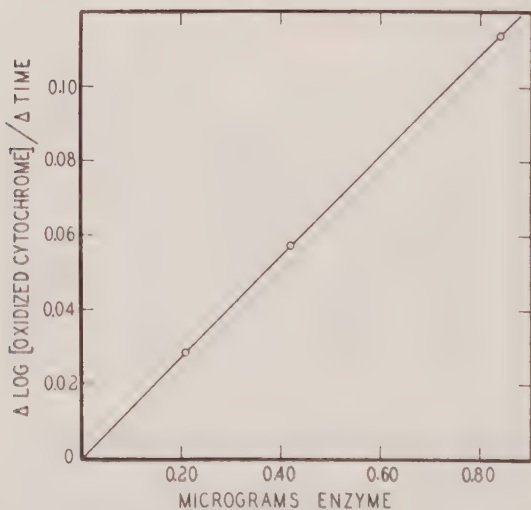


FIG. 5. Rate of reduction of cytochrome *c* as a function of reductase concentration.

2 valence change, we may tentatively postulate that the reductase forms an intermediary semiquinone-like free radical in the course of the reaction. This conclusion is not surprising in view of the finding of Haas that such a compound was observable for the "old" yellow enzyme (17).

From the data in Table IV we are able to calculate minimum values for the specific reaction velocities for the oxidation of cytochrome *c* reductase by cytochrome *c* and also for the reduction of the reductase by dihydrotriphosphopyridine nucleotide. In the first case we assume that all the reductase is in the reduced form; in the second case we assume that the reductase is all in the oxidized form. As these values for the oxidized and reduced

TABLE V
Specific Reaction Velocities at 25°

$K = \text{min.}^{-1} \text{ mole}^{-1} \text{ liter.}$

Enzyme	Prosthetic group	Reaction with dihydrotriphosphopyridine nucleotide, K (reduced) $\times 10^{-5}$	Reaction with cytochrome <i>c</i> , K (oxidized) $\times 10^{-5}$	Reaction with oxygen, K (oxidized) $\times 10^{-5}$
Cytochrome <i>c</i> reductase	Alloxazine mono-nucleotide	>520	>280	
"Old" yellow enzyme	" "	60	0.3	1
"New" yellow enzyme	Alloxazine dinucleotide	220	0	0.14

concentrations of the reductase are maximum values, we obtain only minimum values for the reaction constants. In Table V we compare these values with the known or calculable values for reactions involving the "old" and the "new" yellow enzymes (2). The constant for the reaction between "old" yellow enzyme and cytochrome *c* is based upon data obtained with our own preparation. We have also included the specific reaction velocity constants for the reactions between the "old" and "new" yellow enzymes and oxygen in order to show the relative rates of oxidation by cytochrome and by oxygen. We have not yet determined whether the reduced form of our enzyme can be readily oxidized by oxygen but the evidence is definite that this enzyme is much

more active toward triphosphopyridine nucleotide and cytochrome *c* than either of the other two enzymes. The reduction of cytochrome *c* by the "old" yellow enzyme as reported by Theorell (3) can easily be explained by the assumption that preparations of this enzyme contained only 1 part per thousand of active cytochrome *c* reductase.

Prosthetic Group

In the following we shall demonstrate that the prosthetic group of cytochrome *c* reductase is alloxazine mononucleotide, the same prosthetic group as that associated with the "old" yellow enzyme. To do this we shall describe experiments in which the prosthetic groups of cytochrome *c* reductase, of the "old" yellow enzyme, and of the amino acid oxidase are interchanged. We shall also substantiate our conclusion by a report of a phosphorus determination on the prosthetic group of the reductase.

Splitting and Resynthesis of Cytochrome c Reductase

The reductase can be split and separated into a flavin and a colorless protein in the presence of a high hydrogen ion concentration. The method of effecting this splitting was first suggested by Warburg and Christian (18). Each part of the dissociated enzyme is by itself inactive but by combining the two the active enzyme can be resynthesized.

The prosthetic group was prepared by dissolving 70 mg. of enzyme (obtained from step (5), purity = 0.32) in 8 cc. of water at 0°. To this solution were added 2 gm. of $(\text{NH}_4)_2\text{SO}_4$ and 2 cc. of $\text{N H}_2\text{SO}_4$. The pH of the resulting solution was 2.3 and the degree of saturation was 0.35 with respect to $(\text{NH}_4)_2\text{SO}_4$. The precipitated protein was separated by centrifugation and discarded. The supernatant solution which contained the flavin was neutralized with 0.50 cc. of 2 N KOH. We thus obtained 9.0 cc. of a clear yellow solution. The flavin in this solution we shall designate henceforth as flavin_{c.r.}. Spectrophotometrically this solution was found to contain 0.40×10^{-7} mole of flavin per cc.

To obtain the active protein component 8.8 mg. of an enzyme preparation (purity = 0.62) were dissolved in 6 cc. of a solution which was 35 per cent saturated with $(\text{NH}_4)_2\text{SO}_4$, at 0°. 0.8 cc. of a solution which was 35 per cent saturated with $(\text{NH}_4)_2\text{SO}_4$

and was 0.1 N with respect to H_2SO_4 was then added so that Congo paper was just blue. The protein was precipitated and separated by centrifugation. It was then washed with 3 cc. of a 50 per cent saturated $(\text{NH}_4)_2\text{SO}_4$ solution and dissolved in 4 cc. of 0.05 M phosphate buffer, pH 7.3. 1 cc. of this colorless solution contained 1.5 mg. of protein which we shall designate as protein_{c.r.}; purity 0.50, yield 37 per cent.

The activity of each of the components and of the resynthesized enzyme was determined by means of the test previously described

TABLE VI
Enzymatic Activity of Separated and Combined Enzyme Components

Protein _{c.r.} = 0.92 γ per cc.			Flavin _{c.r.} = 2.5×10^{-11} mole per cc.			Protein _{c.r.} = 0.92 γ per cc. Flavin _{c.r.} = 2.5×10^{-11} mole per cc.		
<i>t</i>	$\frac{8 + \log (\text{CyFe}^{+++})}{\Delta t}$	$\frac{\Delta \log (\text{CyFe}^{+++})}{\Delta t}$	<i>t</i>	$\frac{8 + \log (\text{CyFe}^{+++})}{\Delta t}$	$\frac{\Delta \log (\text{CyFe}^{+++})}{\Delta t}$	<i>t</i>	$\frac{8 + \log (\text{CyFe}^{+++})}{\Delta t}$	$\frac{\Delta \log (\text{CyFe}^{+++})}{\Delta t}$
<i>min.</i>			<i>min.</i>			<i>min.</i>		
0	0.710		0	0.734		0	0.720	
1	0.698	0.012	1	0.731	0.003	1	0.633	0.087
2	0.684	0.013	2	0.728	0.003	1.5	0.606	0.076
						2	0.570	0.075
Average for $\frac{\Delta \log (\text{CyFe}^{+++})}{\Delta t}$ = 0.012			0.003			0.079		

Protein_{c.r.} = protein of cytochrome reductase; flavin_{c.r.} = flavin of cytochrome reductase.

(Table IV). In Table VI the activities of flavin_{c.r.}, protein_{c.r.}, and flavin_{c.r.} + protein_{c.r.} are compared.

This experiment shows clearly that the activity is restored when the two components are recombined. The small activity of the protein alone is probably due to some unsplit enzyme in the protein solution.

Dissociation Constant

To determine the constant for the dissociation of cytochrome *c* reductase into the prosthetic group and protein we added the

flavin in different concentrations to a small but constant amount of the protein at pH 7.3 and at 25°. The activity of the resynthesized enzyme was determined as usual (Table IV). For these determinations only 0.9 γ of protein and about 3×10^{-3} γ of flavin are necessary; this makes available a method for determining very small amounts of alloxazine mononucleotide. The results of this experiment are given in Fig. 6. Since the dissociation constant for this flavoprotein is very small, a considerable part of the added flavin is bound to the protein; thus the dissociation constant is not equal to the total flavin concentration when

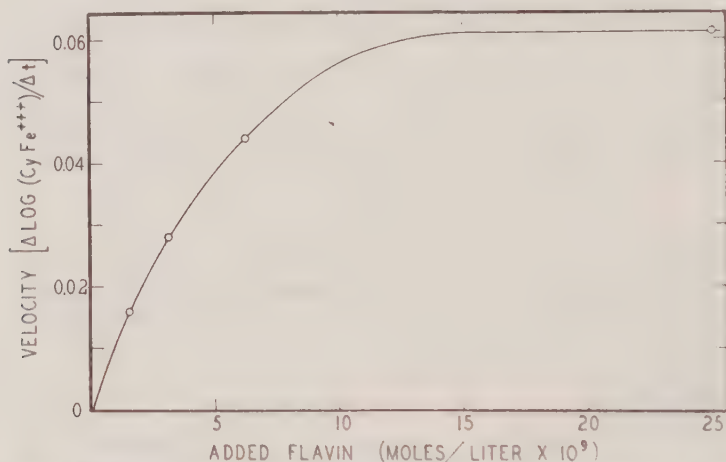


FIG. 6. Rate of reduction of cytochrome *c* as a function of flavin concentration. Increasing concentrations of flavin_{cr} added to 0.92 γ of protein_{cr}.

the activity is 50 per cent that of maximum. Rather two points on the curve must be used and the constant determined by the solution of simultaneous equations.

The value of the dissociation constant K can be determined from the following considerations. Consider two points on the curve, points (1) and (2). Let V_1 be the rate corresponding to point (1) and V_2 the rate for point (2). V_{max} is the maximum rate determined with excess flavin. P_t = total protein concentration, P_1 and P_2 = free protein, E_1 and E_2 = enzyme concentration, F_{t1} and F_{t2} = total flavin, and F_1 and F_2 = free flavin at points (1) and (2) respectively.

$$\begin{aligned}
 P_t &= E_1 + P_1 = E_2 + P_2 \\
 F_{t1} &= F_1 + E_1; F_{t2} = F_2 + E_2 \\
 E_1 &= P_t \frac{V_1}{V_{\max.}}; E_2 = P_t \frac{V_2}{V_{\max.}} \\
 K &= \frac{P_1 \times F_1}{E_1} = \frac{P_2 \times F_2}{E_2}
 \end{aligned}$$

It can then be shown that

$$P_t = \frac{\left(\frac{V_{\max.} - V_1}{V_1} \times F_{t1} \right) - \left(\frac{V_{\max.} - V_2}{V_2} \times F_{t2} \right)}{\frac{V_2 - V_1}{V_{\max.}}}$$

With P_t known, the value of K can readily be calculated. The two upper points on the curve were used in this determination, since the experimental errors are less than those for the lowest activity. The value of the constant so determined is 1×10^{-9} mole liter⁻¹. Since the rate of the reaction is limited by the triphosphopyridine nucleotide concentration, it is assumed that most of the flavoprotein is in the oxidized state during the course of the reaction. The dissociation constant is therefore that for the oxidized form.

By the same sort of calculation we have determined the dissociation constants for the "old" and "new" yellow enzymes. These values together with those for the cytochrome reductase and amino acid oxidase are as follows:

Cytochrome <i>c</i> reductase.....	1×10^{-9}	mole	$\times$	liter ⁻¹
"New" yellow enzyme.....	27×10^{-9}	"	$\times$	"
"Old" " " ".....	60×10^{-9}	"	$\times$	"
Amino acid oxidase.....	250×10^{-9}	"	$\times$	"

The value for the dissociation constant of the amino acid oxidase was determined by Warburg and Christian (18). The cytochrome reductase is considerably more stable with respect to dissociation than are these other flavoproteins.

Prosthetic Group of Cytochrome c Reductase Exchanged with That of "Old" Yellow Enzyme

A preparation of "old" yellow enzyme was made by Mr. H. Persky of this laboratory according to the directions given by

Warburg and Christian (1). The enzyme was further purified by adsorption on aluminum hydroxide gel and subsequent elution with $(\text{NH}_4)_2\text{SO}_4$, a method first suggested by Kuhn and Sörensen (19).

The prosthetic group of the "old" yellow enzyme, alloxazine mononucleotide, was obtained by following the directions of Theorell (13).

To obtain the protein component, it was necessary to purify the enzyme to a greater extent than for the preparation of the prosthetic group. This was carried out by adsorption with $\text{Ca}_3(\text{PO}_4)_2$.

4 cc. of the "old" yellow enzyme as previously prepared (3.6×10^{-7} mole) were dialyzed for 2 days. The enzyme was then adsorbed on 250 mg. of $\text{Ca}_3(\text{PO}_4)_2$ gel. After centrifugation the calcium phosphate with the adsorbed enzyme was washed with 0.05 M phosphate buffer, pH 6.1. The enzyme was then eluted twice with 3 cc. of alkaline $(\text{NH}_4)_2\text{SO}_4$.

The protein was separated from the prosthetic group by the method of Warburg and Christian (18) which involves the splitting with acid in ammonium sulfate solution.

Prosthetic Group of "Old" Yellow Enzyme Replacing the Prosthetic Group of Cytochrome c Reductase—To determine whether the prosthetic group of cytochrome c reductase is alloxazine mononucleotide the prosthetic groups of both the "old" yellow enzyme and of the reductase were added to an excess of the reductase protein. Under these conditions the reaction velocity is a function of the flavin concentration. The activities of the two flavins were compared by the test previously described (Table IV). The results are given in Table VII.

These results show that the activities of the two prosthetic groups are quantitatively the same and that in all likelihood the prosthetic group of cytochrome c reductase is alloxazine mononucleotide.

Prosthetic Group of Cytochrome c Reductase Replacing the Prosthetic Group of "Old" Yellow Enzyme—In the following experiment the prosthetic groups of both yellow enzymes are combined with the protein of the "old" yellow enzyme. The activities of these synthesized enzymes were determined manometrically under the conditions described by Theorell (13). The results are apparent in Table VIII.

In Experiments 3 and 4 of Table VIII it is again demonstrated that the activities of the two prosthetic groups are the same within experimental error, and that they are very probably identical. The determination reported in Experiment 5 was made to demonstrate that excess protein was present; that the flavin determines the rate of oxygen uptake.

*Prosthetic Group of Cytochrome *c* Reductase Replacing That of *d*-Amino Acid Oxidase*

The previous experiments leave little doubt that the prosthetic group of cytochrome *c* reductase is alloxazine mononucleotide.

TABLE VII
Activity of Flavins

Protein_{cr.} = 0.92 γ per cc.

Flavin of cytochrome reductase, 0.31 $\times 10^{-11}$ mole per cc.			Flavin of "old" yellow enzyme, 0.31 $\times 10^{-11}$ mole per cc.		
<i>t</i>	8 + log(CyFe ⁺⁺⁺)	$\frac{\Delta \log(\text{CyFe}^{+++})}{\Delta t}$	<i>t</i>	8 + log(CyFe ⁺⁺⁺)	$\frac{\Delta \log(\text{CyFe}^{+++})}{\Delta t}$
<i>min.</i>			<i>min.</i>		
0	0.710		0	0.720	
1	0.663	0.047	1	0.675	0.045
2	0.625	0.043	2	0.636	0.042
3	0.583	0.042	3	0.595	0.042
4	0.542	0.042	4	0.551	0.040
5	0.490	0.044	5	0.528	0.041
Average.....		0.044			0.042

Nevertheless we tested it in the following system which is specific for alloxazine-adenine dinucleotide, the prosthetic group of *d*-amino acid oxidase. Alloxazine-adenine dinucleotide was prepared by Mr. B. Block of this laboratory according to the directions given by Warburg and Christian (18). The protein component of the amino acid oxidase was made in accordance with the procedure of Negelein and Brömel (14). The results of these experiments are contained in the Table IX.

In every test we have found the prosthetic group of cytochrome *c* reductase to have the same activity as alloxazine mononucleotide. The results of Table IX show clearly that it is not alloxazine-adenine dinucleotide.

TABLE VIII
Replacement of Prosthetic Group of "Old" Yellow Enzyme by That of Cytochrome Reductase
Protein = protein of "old" yellow enzyme; 38°; cup, 0.1 cc. of 2 N KOH; gas space, oxygen.

	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5
	2.6 cc. water 18 mg. hexose monophosphate (K salt) 2 mg. <i>Zwischenferment</i> 0.52 mg. KCN 0.08 " triphosphopyridine nucleotide 1.0 mg. protein	→	, →	→	→
		2×10^{-9} mole flavin _{c.r.}	1.0 mg. protein 2×10^{-9} mole flavin _{c.r.}	1 mg. protein 2×10^{-9} mole alloxazine mononucleotide	1 mg. protein 4×10^{-9} mole alloxazine mononucleotide
C.mm. O ₂ taken up in 10 min.	2	1.5	38	42	74

TABLE IX
Prosthetic Group of Cytochrome c Reductase Replacing That of d-Amino Acid Oxidase
 Protein = protein of d-amino acid oxidase; 38°; cup, 0.1 cc. of 2 N KOH; gas phase, oxygen.

	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5
	2.6 cc. 0.04 M pyrophosphate buffer, pH 8.1 0.47 mg. protein 9.0 mg. <i>D</i> -alanine	→ 1.5 × 10 ⁻⁹ mole flavin _{c.r.}	→ 1.5 × 10 ⁻⁹ mole alloxazine mononucleo- tide	→ 1.5 × 10 ⁻⁹ mole alloxazine- adenine di- nucleotide	→ 3.0 × 10 ⁻⁹ mole alloxazine- adenine di- nucleotide
C.mm.O ₂ taken up in 10 min.	6	20	19	122	210

Determination of Phosphorus Content of Prosthetic Group

To confirm the conclusion that the prosthetic group is alloxazine mononucleotide, we determined the number of phosphorus atoms per molecule of flavin_{c.r.}. In alloxazine mononucleotide this number is 1 and in the dinucleotide it is 2.

The phosphorus determination was carried out according to the following procedure. To 2.0 cc. of the solution containing 0.668×10^{-7} mole of enzyme, 0.1 cc. of 40 per cent trichloroacetic acid was added. Under these conditions the enzyme is split and the protein precipitated. After centrifugation the protein was washed with 2 cc. of 2 per cent trichloroacetic acid and discarded. The supernatant and the wash solution which contain the prosthetic group were combined and evaporated to dryness. The residue was digested with 1.0 cc. of 10 N H_2SO_4 and a drop of perhydrol. Water, molybdate, and sulfonic acid reagent were added in accordance with the procedure given in the following paper (20). The blue phosphomolybdate complex was developed by heating to 100° and from the light absorption at $670 \text{ m}\mu$ ($I_0/I = 2.47$) in a 1.98 cm. cell the amount of phosphorus was calculated to be 2.5 γ .

The 2.5 γ of phosphorus correspond to 0.80×10^{-7} mole of phosphorus. The proportion of phosphorus to flavin = $0.80/0.668 = 1.2$. In view of the fact that the enzyme had been treated with phosphate during preparation, it is understandable that this ratio could be somewhat high, but this result together with all the foregoing experiments indicates that the prosthetic group contains 1 phosphate atom per molecule and is alloxazine mononucleotide.

DISCUSSION

The very high activity of cytochrome *c* reductase both in oxidizing reduced triphosphopyridine nucleotide and in reducing oxidized cytochrome *c* makes it seem highly probable that this enzyme constitutes a missing link in the oxidation-reduction chain. Although the potential of cytochrome *b* lies between that of cytochrome *c* and the flavoproteins, there is now no necessity to include it in this part of the oxidation mechanism. The enzymatic functions of both cytochromes *a* and *b* are therefore still left to be elucidated.

The fact that the prosthetic group of cytochrome *c* reductase is alloxazine mononucleotide places this coenzyme in a more significant position as a biologically important compound. The only

other known flavoprotein having this same prosthetic group is the "old" yellow enzyme.

In view of the unstable nature of cytochrome *c* reductase it is now easy to understand the reason that this substance previously escaped detection. If cytochrome *c* reductase is subjected to the same treatment as that used in the isolation of other flavoproteins, its activity toward cytochrome *c* is destroyed. In the preparation of the "old" yellow enzyme Warburg and Christian kept their product in 33 per cent acetone for 24 hours. Under these conditions cytochrome *c* reductase loses its activity completely in less than 1 hour. Another of their purification steps involves shaking for 24 hours at 38°. The activity of our enzyme in reducing cytochrome *c* is diminished to the extent of 37 per cent when kept for 10 minutes at 40°. Since we have not yet studied the reaction between the reductase and molecular oxygen, we are not in a position to make any statement regarding the stability of our enzyme in its reaction with oxygen. Inasmuch as the free flavin is autoxidizable, it is conceivable that the activity of our enzyme toward cytochrome *c* may be destroyed while that toward oxygen may not.

SUMMARY

1. A new flavoprotein, cytochrome *c* reductase, which completes the oxidation-reduction chain between hexose monophosphate and cytochrome *c* is reported.

2. A method for isolating this enzyme from yeast is described.

3. The molecular weight of the enzyme was determined to be about 75,000. The purity of the final product was found to be about 87 per cent.

4. The absorption spectrum and the method of titrating cytochrome *c* reductase by cytochrome *c* are described.

5. The analytical method, based upon spectrophotometric rate determinations, is given, together with a somewhat general treatment of the kinetics involved in the test. Minimum values of the rate constants are determined and it is demonstrated that cytochrome *c* reductase reacts specifically with cytochrome *c*, whereas the "old" or "new" yellow enzymes do not.

6. The enzyme can be split into a protein and a prosthetic group which is alloxazine mononucleotide. By recombining these the enzyme can be resynthesized.

7. The evidence that the prosthetic group is alloxazine mononucleotide is given by the interchange of the prosthetic groups of cytochrome *c* reductase, the "old" yellow enzyme, and the amino acid oxidase. This is supported by a determination of the phosphorus in the prosthetic group.

8. The constant for the dissociation of the oxidized form of the enzyme into its protein and alloxazine mononucleotide is determined to be 1×10^{-9} mole liter⁻¹. This enzyme is considerably less dissociated than any of the other yellow enzymes.

9. The enzyme is very unstable with respect to low pH and to denaturation by heat.

10. A method is given for determining very small amounts (10^{-3} γ) of alloxazine mononucleotide.

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